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Systemic Transforming Growth Factor-Beta in Patients With Bone Marrow Fibrosis—Pathophysiological Implications

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Idiopathic myelofibrosis (IMF) and secondary myelofibrosis (MF) are characterized by bone marrow (BM) fibrosis, neoangiogenesis, and increased extracellular matrix (ECM) proteins. These characteristics may be partially attributed to transforming growth factor beta (TGF-β), a cytokine produced by monocytes. In myelofibrosis, monocytes are increased and activated with concomitant up-regulation of intracytoplasmic TGF-β. We have therefore determined systemic TGF-β in patients with either BM fibrosis: IMF, n = 18; MF, n = 16; or without BM fibrosis: hematologic disorders with normal platelets (n = 31); high platelets (n = 9); or normal controls (n = 27). Compared with nonfibrosis sera, there was significant TGF-β elevation in BM fibrosis sera ($P < 0.0001$). Most (>80%) of the TGF-β is active and belongs to the −β1 isoform. In situ hybridization and immunohistochemical analyses in BM biopsy sections showed a marked increase in TGF-β1 only in patients with fibrosis. Moreover, TGF-β protein was detected mainly in myelomonocytic-like predominant areas. To determine if another functionally similar cytokine, basic fibroblast growth factor (bFGF), may be important to BM fibrosis, we quantitated sera levels and found elevation in 57% compared with 100% elevation for TGF-β. The data indicate that irrespective of etiology, systemic TGF-β is elevated in patients with BM fibrosis. TGF-β likely plays an important role in the development of BM fibrosis. The study also provides a significant parameter for early therapeutic intervention in BM fibrosis. Am. J. Hematol. 59:133–142, 1998. © 1998 Wiley-Liss, Inc.

Key words: transforming growth factor; basic fibroblast growth factor; bone marrow; fibrosis; myelofibrosis

INTRODUCTION

Idiopathic myelofibrosis (IMF) and secondary myelofibrosis (MF) are disorders characterized by: 1. hypercellularity [1]; 2. excessive deposits of extracellular matrix (ECM) proteins in the bone marrow (BM) [1]; 3. high levels of ECM proteins in the circulation [2]; 4. extramedullary hematopoiesis [3–5]; 5. BM neoangiogenesis [6]; and 6. increased fibroblasts in the BM [7–9]. Advanced myelofibrosis is associated with impaired hematopoiesis partially characterized by leukoerythroblastosis in the peripheral blood. Several disorders underlie the development of BM fibrosis. These include AIDS, lymphomas, multiple myeloma, acute and chronic leukemias, metastatic solid tumors, other myeloproliferative disorders, and autoimmune diseases such as systemic lupus erythematosus [3,10–13]. In patients with myelofibrosis, whereas there is no evidence of clonality in BM fibroblasts, there is clonal expansion of the hematopoietic progenitors [1,3,14–16]. This has led to the suggestion that enhanced proliferation of BM fibroblasts is a reactive process mediated by the clonal cells [17–21]. Immune-mediated mechanisms have also been implicated in the pathophysiology of BM fibrosis [22,23].

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Specific immune and hematopoietic cells, macrophages and megakaryocytes, increased in the BM of myelofibrosis, are implicated in the development of BM fibrosis [24–26]. Peripheral blood monocytes from IMF are activated [27]. This state of activation may provide immune-related parameters relevant to the development of BM fibrosis.

Secondary immune-mediators such as platelet-derived growth factor, transforming growth factor-beta (TGF-β), macrophage-colony stimulating factor, and interleukin-1 (IL-1), have been associated with the pathogenesis of BM fibrosis [24,28,29]. The experimental evidence suggests that TGF-β may be central to mechanisms leading to the development of BM fibrosis. In fact, circulating IMF monocytes are activated and contain increased intracellular TGF-β [27]. In vitro studies have shown that interactions between monocytes and ECM proteins lead to enhanced production of TGF-β [30]. Furthermore, the biological functions of TGF-β are consistent with the pathological profile of BM fibrosis [1,2,6]. In addition, a role for TGF-β in BM fibrosis is supported by the experimental data [27,30]. TGF-β is mitogenic to mesenchymal cells, induces angiogenesis and ECM proteins, and inhibits the breakdown of collagen [31,32]. The latter function will lead to the accumulation of collagen, evident in patients with myelofibrosis [1].

We hypothesize that the expression of TGF-β and, to a lesser extent, bFGF, are enhanced in patients with BM fibrosis. The rationale being that TGF-β can be overproduced through interactions among adhesion molecules, expressed on immune and hematopoietic cells, and ECM proteins [27,30], ubiquitously present in myelofibrosis patients. Furthermore, megakaryocytes and monocytes, sources of TGF-β, are increased in patients with BM fibrosis [25,33]. In this study, we have determined systemic TGF-β levels in patients with BM fibrosis by: 1. quantitation in the sera; 2. steady state TGF-β1 messenger ribonucleic acid (mRNA) levels in BM biopsies by in situ hybridization; and 3. relative TGF-β densities in BM biopsies by immunohistochemistry. Comparisons were made with patients that had other hematologic disorders without fibrosis (HC) and healthy normal controls (NC). The cellular sources of TGF-β in myelofibrosis remain unclear. Whereas we reported that myelofibrosis monocytes are sources of TGF-β [27], others reported that megakaryocytes may be the likely source [24,26]. Because platelets are major sources of TGF-β and they are increased in many myelofibrosis patients [26], we have included a group with no fibrosis, but comparable to the platelet counts of patients with myelofibrosis (HC-hPl). Circulating TGF-β levels in the HChPl group may help to unravel questions regarding the major cellular sources of TGF-β in patients with BM fibrosis. We have found that despite the etiology, active TGF-β is increased in the sera of patients with BM fibrosis. In situ hybridization and immunohistochemistry also indicate increase in TGF-β1 mRNA and protein in myelomonocytic-like areas of BM biopsy sections. TGF-β mRNA was not detected in the fibroblast-rich areas suggesting that they may be unlikely contributors of TGF-β in the BM. We have also found that most of the TGF-β in the circulation is active and that the major isoform is −β1.

METHODS

Study Subjects

This study was performed in accordance with the Institutional Review Boards of UMDNJ-New Jersey Medical School and East Orange Veterans Administration Medical Center. Study subjects included: NC (n = 27); IMF (n = 18); MF (n = 16); HC (n = 31); and HC-hPl (n = 9). The MF group included the following patients: chronic myelogenous leukemia (n = 7); polycythemia vera (n = 3); essential thrombocythemia (n = 2); myelodysplastic syndrome (n = 2); non-Hodgkin's lymphoma (n = 1); and systemic lupus erythematosus (n = 1). HC patients included: aplastic anemia (n = 3); non-Hodgkin's lymphoma (n = 3); mycosis fungoides (n = 2); Hodgkin's lymphoma (n = 1); acute myelogenous leukemia (n = 5); acute lymphoblastic leukemia (n = 3); chronic lymphocytic leukemia (n = 3); T-cell leukemia/lymphoma (n = 1); myelodysplastic syndrome (n = 5); polycythemia vera (n = 2); multiple myeloma (n = 1); agranulocytosis (n = 1); and systemic mastocytosis (n = 1). HC-hPl included essential thrombocythemia (n = 5) and reactive thrombocytosis (n = 4). The mean platelet count of the HC-hPl group was equivalent to 1.2 ± 0.4 × $10^6/\mu l$ (±SD).

The ages of the study populations ranged from 36 to 78 years (Table I). The mean ages (years) were as follows: IMF = 64; MF = 59; HC = 61; HC-HPl = 65; and NC = 51. Patients with MF had moderate-to-significant fibrosis by BM biopsy. At diagnosis, 81% of the IMF patients had a dry tap BM aspiration. Diagnosis was based on established clinical and laboratory criteria. At the time of participation, subjects were neither infected, nor were they taking immunosuppressive agents or medications. Because of our exclusion criteria, this study was conducted during a 10-year period.

Sera and/or plasmas were separated from peripheral blood obtained from study subjects. Samples were aliquoted in siliconized tubes and then stored at −70°C until ready to be assayed for TGF-β and bFGF. Samples were thawed only once. Studies with BM biopsy sections were performed with paraffin-embedded tissues that were obtained from the pathology archives. Except for the clinical record, tissues from either patients with BM fibrosis (IMF) or without fibrosis (HC) were obtained anonymously.

TABLE I. Hematologic Indices on Patients With IMF*

Patient	Age/Sex	Hb (g/dL)	WBC ($\times 10^9$/L)	Platelets ($\times 10^9$/L)
1	71/F	6.2	3.6	79
2	38/F	4.6	2.9	58
3	66/F	9.1	21.9	524
4	70/M	11.9	5.5	69
5	66/F	8.1	19.9	158
6	48/F	7.1	2.3	40
7	36/F	12	4.0	124
8	66/M	9.9	26.3	102
9	71/M	8.5	6.5	175
10	69/M	10	5.4	356
11	72/M	9.4	13.5	487
12	56/M	10.5	48	640
13	76/F	4.2	5.9	49
14	68/M	8.4	6.1	310
15	76/F	6.8	5.7	45
16	61/M	7.8	5.2	220
17	70/M	6.7	11.7	41
18	78/M	11.4	9.3	125

*IMF, idiopathic myelofibrosis; Hb, hemoglobin; WBC, white blood. Levels of Hb, WBC, and platelets were determined on the day that sera were collected from patients with IMF. Normal hematologic indices are as follows: Hb, 12–16 g/dL; WBC, (4.5–11) × 10^9/L; platelets, (140–400) × 10^9/L.

Reagents

Proteinase K, ribonuclease (Rnase), paraformaldehyde, and diethylpyrocarbonate (DEPC) were purchased from Sigma (St Louis, MO). Phosphate buffered saline (PBS), pH 7.4, was purchased from Cellgro, Mediatech (Herndon, VA). Formamide was purchased from Life Technologies (Grand Island, NY).

Determination of TGF-β Levels

TGF-β bioassay is based on the growth inhibition of the mink lung epithelial cells (CCL 64) as described [27]. Each sample was tested in triplicate with three different undiluted volumes: 25 μl, 50 μl, and 100 μl. TGF-β levels were determined from a standard curve established with TGF-β concentrations ranging from 0.001 to 10 ng/ml. Standard TGF-β was purchased from R&D Systems, Minneapolis, MN. For each sample with ⩾20 ng/ml TGF-β, the assay was repeated in the presence or absence of neutralizing rabbit polyclonal anti-hTGF-β (R&D Systems). This antibody exhibits specificity for −β1 isoform. Therefore, the levels of TGF-β neutralized in the bioassay may be TGF-β1 isoform.

The CCL 64-cell assay does not detect latent TGF-β. To determine total TGF-β levels (latent plus active), we activated the latent form. This was accomplished by acidification as described [34]. Samples were incubated with 1N HCl for one hr at 4°C followed by neutralization with 0.2 N NaOH. During pH adjustment, samples were diluted ¼ and this dilution factor was corrected in the final calculations. To verify that the latent TGF-β was totally activated, we took advantage of the fact that the antibody binds to the latent (100 kDa) and active forms (25 kDa). Western blots (see below for method), lacking reducing agent indicated bands at 25 kDa for acidified samples. For untreated samples, bands at 25 and 100 kDa were observed (data not shown). Immunoreactive TGF-β and bFGF were quantitated by direct enzyme linked immunosorbent assay (ELISA) [35], and sandwich ELISA (Quantikine kit, R&D Systems), respectively.

Immunoblots

Sera (one ml) from one of the following: IMF (n = 3); MF (n = 2); HC (n = 1); and NC (n = 1) were cleared of immunoglobulin G (IgG). This was accomplished by overnight incubation at 4°C with 0.01 g protein A sepharose CL 4B (Sigma). Protein A was removed by centrifugation at 4,000g for five min and sera dialyzed (6,000–8,000 MW cut off) overnight at 4°C in 0.02 M phosphate buffer. Sera were cleared further of albumin by column chromatography consisting of affi-gel blue 50–100 mesh (Bio Rad, Hercules, CA). Eluates were concentrated to the original starting volume with Centriprep 10 microconcentrator (Amicon, Danvers, MA). To prevent nonspecific adherence of TGF-β, samples were fractionated in siliconized tubes. Protein concentrations, based on the Bradford procedure (Bio Rad), before and after fractionation were 21 ± 6 mg/ml ($\bar{x}$ ± SD) and 1.8±0.2 mg/ml ($\bar{x}$ ± SD), respectively.

Latent TGF-β in the partially fractionated samples was activated as described above. For each activated sample, 40 μg of total proteins were analyzed by Western blots on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions as described [27]. Immunoblotting was performed with optimal concentration of rabbit polyclonal anti-hTGF-β, one ng/ml. The molecular weights of developed bands were compared with prestained midrange standards (Diversified Biotech, Newton Centre, MA). Optimal anti-hTGF-β was predetermined in Western blots with various concentrations of TGF-β and antibody ranging from two to 0.5 ng/ml. We determined that one ng anti-hTGF-β detected TGF-β ranging from 10 to 0.005 ng.

In Situ Hybridization

Paraffin-embedded BM biopsy tissues from either IMF or HC were sectioned into 4–6 microns with a Leica rotary microtome (Leica Instruments, Nussloch, Germany). Sections were placed on Superfrost/Plus slides (Fisher Scientific, Springfield, NJ) and then deparaffinized as follows: consecutive washing in xylene (2 × 5 min); 99% ethanol (2 × 1 min); 95% ethanol (2 × 1 min); and DEPC-treated water (1 × 5 min). Sections were studied for TGF-β1 mRNA by in situ hybridization and TGF-β protein by immunohistochemistry (see below).

Before hybridization, sections were cleared of proteins by digesting with proteinase K (30 μg/ml) at 37°C for one hr. Negative control slides were subjected to treatment with RNase (100 (μg/ml) for 30 min at 37°C. Enzymatic reactions were stopped by post-fixing with 0.4% paraformaldehyde in PBS. Slides were prehybridized for one hr at 37°C. Prehybridization solution contains equal volumes of commercially derived solution (5 Prime→3 Prime (Boulder, CO) and formamide. Salmon sperm, 10 mg/ml (5 Prime→3 Prime) was used as blocking DNA during prehybridization. Slides were drained and then hybridized for 18 hr at 37°C with 200 ng/ml oligonucleotide probes. Hybridization probes consisted of a cocktail of three digoxigenin-labeled oligonucleotide probes specific for TGF-β1 mRNA (R&D Systems). During hybridization, slides were covered with siliconized (Sigmacote, Sigma) cover slips. After hybridization, sections were washed as follows: 4 × sodium chloride/sodium citrate (SSC) buffer/30% formamide, followed by another wash in two × SSC/30% formamide, and a third wash with 0.2 × SSC/30% formamide. Washes were of five min duration at 37°C.

Hybrids were detected by incubating at room temperature for 30 min with 1.2 U/ml alkaline phosphatase-conjugated antidigoxigenin Fab fragment (Boehringer Mannheim, Indianapolis, IN). Sections were developed with the 5-bromo-4-chloro-3-indolyl-phosphatase/nitroblue tetrazolium (BCIP/NBT) phosphate substrate system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for two hr at 37°C. Slides were washed, counterstained with Nuclear Fast Red (J.T. Baker Chemical Co., Phillipsburg, NJ), and then mounted with Pro-Texx medium (Baxter Diagnostics, Inc., Deerfield, IL). Slides were examined with an Olympus B ×40 microscope (Olympus, Lake Success, NY). Except for the negative control (RNase-treated), the labeling technique was performed in RNase-free conditions.

Immunohistochemistry

Tissue sections were deparaffinized for in situ hybridization (see above) and then incubated with one ng rabbit anti-hTGF-β for 16 hr at room temperature. Specificity of the reaction was determined by incubating slides with one ng antibody that was preincubated with 100 pg TGF-β1. Standardization of the staining indicated that the optimum concentration of anti-hTGF-β was similar for immunohistochemistry and Western blot.

After incubation with the primary antibody, sections were next incubated with 100 ng/ml alkaline phosphatase-conjugated goat anti-rabbit IgG (R&D Systems) for two hr at room temperature. Color was developed with the BCIP/NBT substrate system for 30 min. Slides were counterstained with 0.25% methanil yellow (Poly Scientific Research and Development Corp., Bay Shore, NY), mounted, and then microscopically examined for in situ hybridization.

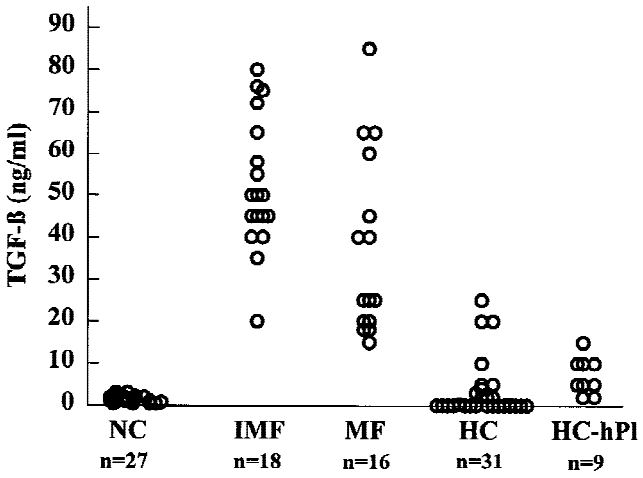

Fig. 1. Circulating levels of active TGF-β in patients with or without BM fibrosis. Sera from NC or patients (IMF, MF, HC, HC-hPl) were quantitated for active TGF-β with the CCL 64 cell as described in Methods.

Statistics

Statistical significance was determined by the Kruskal-Wallis one-way repeated measures, analysis of variance (ANOVA) followed by Tukey's multiple comparison at $P < 0.0001$. All results are given as a two-tailed P value [36].

RESULTS

TGF-β Levels in the Circulation of Patients With or Without BM Fibrosis

We reported previously that adhesion mediates enhanced induction of TGF-β in MF monocytes compared with monocytes from NC [27]. At least one adhesion molecule, CD44, has been implicated in this process [30]. Furthermore, we reported that in MF patients, circulating monocytes are activated with enhanced cytoplasmic TGF-β [27]. ECM proteins can mediate the induction of TGF-β and other fibrogenic cytokines in MF monocytes [30]. We therefore hypothesize that in vivo, cells in contact with ECM matrix proteins could become activated and overproduce TGF-β that could be detected in the circulation. Therefore, we determined TGF-β levels in the sera of IMF, MF, HC, HC-hPl, and NC. Detected TGF-β levels (ng/ml, $\bar{x} \pm$ SE) were as follows: NC, n = 27: 1.4 ± 0.1; HC, n = 31: 3.2 ± 1.2; and HC-hPl, n = 9: 7.1 ± 1.5 (Fig. 1). There was no significant difference in TGF-β levels between NC and HC. However, the mean level in the HC-hPl group was twofold more than in HC with normal platelet counts. MF (37 ± 5, $\bar{x} \pm$ SE, n = 16) and IMF (53 ± 4, $\bar{x} \pm$ SE, n = 18) were not significantly different (Fig. 1) and their levels (IMF and MF, $\bar{x} \pm$ SE: 45 ± 3.4 ng/ml) were statistically significant from patients without fibrosis

(NC, HC, and HC-hPl, $\bar{x}$ ± SE: 3.0 ± 0.6 ng/ml) (P <0.0001).

We next determined immunoreactive TGF-β by ELISA in each patient within the IMF and MF groups. We also determined levels in randomly selected samples that belonged to the NC (n = 3) and HC (n = 7) groups. We determined immunoreactive TGF-β1 (ng/ml, ±SD) in: IMF = 57 ± 6; MF = 46 ± 6; and NC and HC = 3 ± 1. The data, however, indicate statistically significant difference between immunoreactive TGF-β levels in fibrosis and nonfibrosis groups (P <0.0001).

Furthermore, TGF-β has been implicated in the pathogenesis of tissue fibrosis in the lung, liver, and kidney [37,38]. To this end, we further evaluated the biological significance of elevated, circulating TGF-β. We determined TGFβ levels in two patients with severe fibrotic disorders—idiopathic pulmonary fibrosis and generalized scleroderma—and found that their TGF-β levels were 20 and 38 ng/ml, respectively. Interestingly, examination of BM biopsy sections from the patient with generalized scleroderma indicated no fibrosis. The BM status of the other patient is unknown because no biopsy specimen was available. These results suggest that TGF-β levels in nonmyeloproliferative disease and accompanying clinically significant tissue fibrosis can be markedly elevated compared with the nonfibrosis groups (Fig. 1). However, the significance of these results requires TGF-β quantitation in a larger group of nonmyeloproliferative, fibrotic disorders.

Influence of Platelet Degranulation on Sera TGF-β

During sera separation in whole blood, TGF-β can be released from degranulated platelets. Because this study was performed with sera, we determined whether high TGF-β levels in the sera were derived from platelets. This was addressed by determining TGF-β levels in the plasma and sera obtained from the same bleed (n = 8; ng/ml ± SD). We found comparable levels in both types of sample (plasma: 52 ± 6; sera: 56 ± 10). Similar determinations in sera and plasma from the subjects in the HC-hPl group (n = 6; ng/ml ± SD) also indicated comparable TGF-β levels in plasma and sera (plasma: 7.2 ± 1.4; sera: 7.0 ± 1.1). These results suggest that degranulated platelets may be the unlikely source of the high TGF-β levels quantitated in the sera.

TGF-β1 Is the Predominant Isoform in the Sera

The CCL 64 cell assay does not discriminate among the TGF-β isoforms [39]. TGF-β1 is the predominant mammalian isoform [31,32]. We asked whether patients with BM fibrosis might express a different isoform of TGF-β. This was addressed by determining TGF-β1 levels in the sera relative to total TGF-β. Distinct in vivo functions by the various TGF-β isoforms formed the basis of this question [40]. We repeated the TGF-β bioassay in either the presence or the absence of neutralizing polyclonal anti-hTGF-β that has high avidity for the −β1 isoform. To ascertain whether we were in the area of equivalence, we serially diluted each sample of serum and then added a constant antibody concentration (two μg) to each well. We determined that two μg of anti-TGF-β can neutralize 0.01 to five ng of TGF-β1. In each of the IMF and MF samples tested, two μg antibody reversed the inhibitory effects on CCL 64 proliferation. Neutralization occurred in sera dilutions containing TGF-β ranging from 0.01 to 5 ng (Fig. 2). The antibody by itself did not affect CCL 64 cell growth.

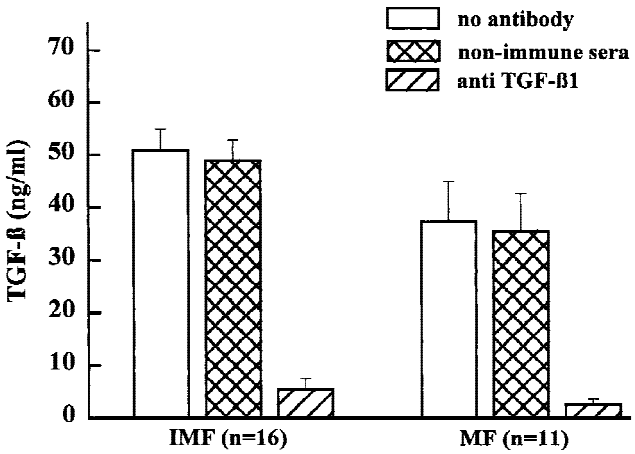

Fig. 2. Inhibition of CCL 64 cell growth is reversed by anti-hTGF-β1. Sera from IMF and MF patients were quantitated for TGF-β in the absence or presence of rabbit nonimmune sera or rabbit polyclonal anti-hTGF-β. TGF-β quantitations were performed with the CCL 64 cell as described in Methods.

To confirm that TGF-β neutralization in the bioassay was not caused by cross reactivity with other sera proteins, we determined the size of the reacting sera proteins by Western blots. We activated the latent TGF-β in partially fractionated sera from either IMF (n = 3), MF (n = 2), HC (n = 1), or NC (n = 1). Western blots were performed by loading constant amounts of partially fractionated total proteins, 40 μg. We observed strong bands in IMF sera (Fig. 3, lanes 1–3) and MF sera (Fig. 3, lanes 6–7). Relatively light bands were observed in NC sera (Fig. 3, lane 4) and HC sera (Fig. 3, lane 8). All bands, including standard TGF-β1 (Fig. 3, lane 5), were observed at 25 kDa. These results indicate that TGF-β1 is the predominant isoform in the sera of patients with BM fibrosis.

Active Versus Latent TGF-β in Sera

The latent form of TGF-β is important in modulating its functions because, in the latent form, transport is more efficient and, therefore, so is its bioavailability [31]. Because the CCL 64 bioassay is sensitive only to active

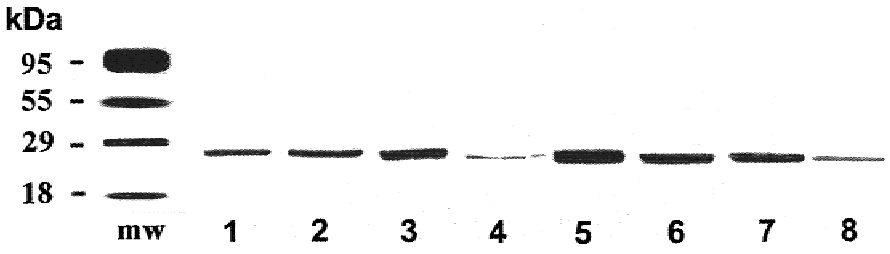

Fig. 3. Western blots for TGF-β1 in partially fractionated sera. Western blots were performed with anti-hTGF-β1 with activated, partially fractionated sera from one of the following: IMF (lanes 1–3); MF (lanes 6–7); HC (lane 8); or NC (lane 4); and 20 ng standard TGF-β (lane 5). All procedures are described in Methods.

TGF-β, quantitation by this cell line does not include the latent form. We therefore determined latent TGF-β levels in the sera of patients with BM fibrosis following acidified activation. These determinations represent the total levels (active and latent). Parallel determinations in untreated samples represent active TGF-β levels. We found that after activation, the mean increase in TGF-β levels was 20% for IMF and MF. For NC, TGF-β levels before and after activation were comparable, <1.0 ng/ml (Table II). The data indicate that although latent TGF-β is present in the circulation of patients with BM fibrosis, the activated form predominates.

TABLE II. Latent and Active TGF-β Levels in Sera of IMF and MF Patients*

	TGF-β Active $\bar{x} \pm$ SD (ng/ml)	TGF-β Latent + Active $\bar{x} \pm$ SD (ng/ml)
NC (n = 20)	<0.05	<0.5
HC (n = 20)	2.5 ± 1	3.0 ± 1
IMF (n = 16)	51 ± 5	65 ± 7
MF (n = 11)	40 ± 6	68 ± 10

*TGF-β, transforming growth factor-beta; IMF, idiopathic myelofibrosis; MF, secondary myelofibrosis; NC, normal controls; HC, hematologic disorders without fibrosis; SD, standard deviation.
Sera obtained from either NC, HC, IMF, or MF were assayed for active TGF-β. In parallel experiments, latent TGF-β in the same samples was activated as described in Methods then assayed for TGF-β (latent plus active). TGF-β levels were determined with the CCL 64 cell line as described in Methods.

Levels of bFGF in Sera of Patients With Bone Marrow Fibrosis

bFGF is mitogenic for fibroblasts and enhances angiogenesis [41]. Also, bFGF, present in the urine of patients with a variety of neoplastic diseases, may contribute to BM fibrosis [42]. We also observed that adhesion induces bFGF mRNA in IMF monocytes (unpublished observations). Sera levels of bFGF were determined in IMF, MF, NC, and HC. Compared with TGF-β in which 100% of the sera assayed were elevated (Fig. 1), only 57% were elevated for bFGF (Fig. 4). bFGF levels (pg/ml ± SE) were: IMF = 12.5 ± 3.2 (n = 16); MF = 8.5 ± 3 (n = 10); NC = 0.5 ± 0.4 (n = 20); and HC = 4.3 ± 1.5 (n = 21). The correlation between BM fibrosis and bFGF is statistically significant (P <0.001), whereas there is no correlation between TGF-β and bFGF levels.

TGF-β1 mRNA and Protein in Bone Marrow Biopsies of Patients With Myelofibrosis

Our previous report demonstrated that circulating IMF monocytes are activated and contain detectable levels of intracytoplasmic TGF-β [27]. Consistent with this report is the compelling evidence that TGF-β may have a physiologic role for BM fibrosis (Fig. 1). Furthermore, compared with bFGF, increased circulating TGF-β levels correlate in all cases with BM fibrosis (Figs. 1 and 4). We next determined whether enhanced TGF-β levels in the sera are similar in the BM. This was addressed by comparing densities of TGF-β1 protein and mRNA in BM tissue sections from patients with (IMF: n = 2; MF: n = 2) or without BM fibrosis (HC: n = 2). The results of both analyses from one IMF and one HC are presented in Figure 5. TGF-β1 protein was detected by immunohistochemistry and its mRNA by in situ hybridization. Subjective grading for HC indicates densities of 0–1 for TGF-β protein (Fig. 5C) and mRNA (data not shown). Densities for both analyses in patients with IMF were 3–4 (Fig. 5D for protein and 5B for mRNA).

Within each tissue section, comparative analysis was made between areas that are predominantly hypercellular and those that consist of spindle-like cells. We observed that TGF-β mRNA is found mostly in the cellular areas. Morphological examination suggested that cells that contain TGFβ mRNA are myelomonocytic-like (Fig. 5B). Cellular morphology was verified by comparing consecutive tissue sections stained with hematoxylin-eosin.

Parallel immunohistochemistry with anti-TGF-β preincubated with TGF-β (complexed antibody) abrogated the ability of the antibody to detect protein (data not shown). These results indicate that positive staining is specific for anti-TGF-β. Similarly, for the in situ technique, slides treated with RNase resulted in negative staining for sections from BM fibrosis (data not shown). This showed that the positive results were not caused by nonspecific-intracytoplasmic binding of the oligonucleotide probes. The results therefore indicate that TGF-β1 mRNA and protein densities are elevated in patients with BM fibrosis compared with HC. The data also suggest that TGFβ may be derived from multiple cellular sources in the BM.

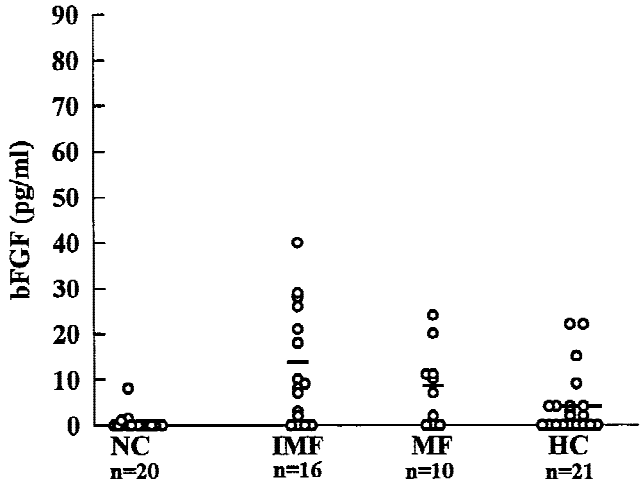

Fig. 4. Immunoreactive bFGF levels in sera of patients with bone marrow fibrosis. bFGF levels were determined in a sandwich ELISA in sera from four study groups: IMF, MF, NC, and HC.

DISCUSSION

BM fibrosis is a hematologic disorder that leads to significant derangement of hematopoiesis. This disorder is associated with marked comorbidity for which the pathogenesis is not understood, and there is no specific treatment. A strong association between TGF-β levels and BM fibrosis has been found despite the underlying etiology (Figs. 1 and 5). Findings of the present study provide a physiologic significance for the activation state of IMF monocytes [27]. The data provide clinical correlation to the in vitro reports in which we showed adhesion-mediated overproduction of TGF-β by myelofibrosis monocytes [27,30]. TGF-β1, being the predominant form and mostly activated in myelofibrosis sera (Table II, Figs. 2 and 3), is consistent with our previous studies. We showed previously that IMF monocytes release mostly active TGF-β [27].

The importance of bFGF remains unclear, although a role in the development of BM fibrosis cannot be discounted. Induction of bFGF could represent a compensation for the elevated levels of TGF-β. bFGF may be up-regulated as an effort to counteract immune suppression and possibly BM dysfunction caused by the high TGF-β levels [43]. Also, the ability of bFGF to induce proliferation of megakaryocyte progenitors [44] may partly account for the megakaryocytosis observed in patients with myelofibrosis. Recent studies indicate that compared with NC, TGF-β and its specific type I receptors are down-regulated in CD34+ cells obtained from patients with MF [45]. However, the opposite is observed for bFGF and its specific receptor [45]. This suggests that bFGF may be more important to the proliferation of the myeloid clone rather than the direct development of BM fibrosis. However, a role for bFGF in the development of BM fibrosis cannot be discounted, although the current data favor a more crucial role for TGF-β.

The development of BM fibrosis may be contributed by potential interactions among TGF-β, other growth factors, and adhesion molecules caused by the complex nature of the fibrogenic process. BM hypercellularity in myelofibrosis may be explained partly by the synergism between increased circulating levels of TGF-β and M-CSF [29]. Furthermore, TGF-β, M-CSF, and CD44, implicated in the pathogenesis of BM fibrosis [30], can prevent cells such as macrophages from undergoing apoptosis [46–48]. Blocking of apoptosis could very well enhance hypercellularity. Increased macrophages in myelofibrosis patients may be caused partly by the ability of TGF-β1 to augment the activity of macrophage differentiation factor, M-CSF, in the BM [25,49]. Furthermore, recent findings indicate that monocytes and BM monocytoid cells are sources of TGF-α [50]. Considering the angiogenic and mitogenic effects on fibroblasts by TGF-α, it is likely that TGF-α may represent another monocyte factor that is important to the development of BM fibrosis.

The sources for circulating TGF-β and bFGF in patients with BM fibrosis are unknown, although megakaryocytes, platelets, and monocytes/macrophages are likely sources [27,51]. Most patients with myeloproliferative disorders, including those with early stages of BM fibrosis, have increased numbers of platelets that contain high levels of TGF-β [26]. However, in this study, the HC-hPl group with very high platelet counts demonstrated low TGF-β levels in their sera (Fig. 1). This suggests that for some patients, platelets may be an unlikely source. In addition, a correlation between BM fibrosis and TGF-β deposition was found to be independent of the number of megakaryocytes [52]. Furthermore, the scleroderma patient included in our study had a low number of megakaryocytes in BM biopsy and no BM fibrosis. The TGF-β level (38 ng/ml) in this patient was comparable to those in myelofibrosis (45 ± 3.4). This suggests that there are other cellular sources for TGF-β in diseases with extensive tissue fibrosis and elevated TGF-β levels. The experimental evidence suggests that monocytes/macrophages could be potential sources of TGF-β in patients with BM fibrosis [30].

This study suggests a strong link between TGF-β and BM fibrosis. In comparison, the data suggest that bFGF is not a sensitive parameter of fibrosis, although a recent report indicated that intracellular bFGF is elevated in patients with BM fibrosis [53]. This report indicates that bFGF appears to be secreted only in patients with the accelerated phase of the disease [53]. This study [53], combined with the data in Figure 4, suggest that bFGF may have a role in the development of BM fibrosis. Because patients with other hematologic

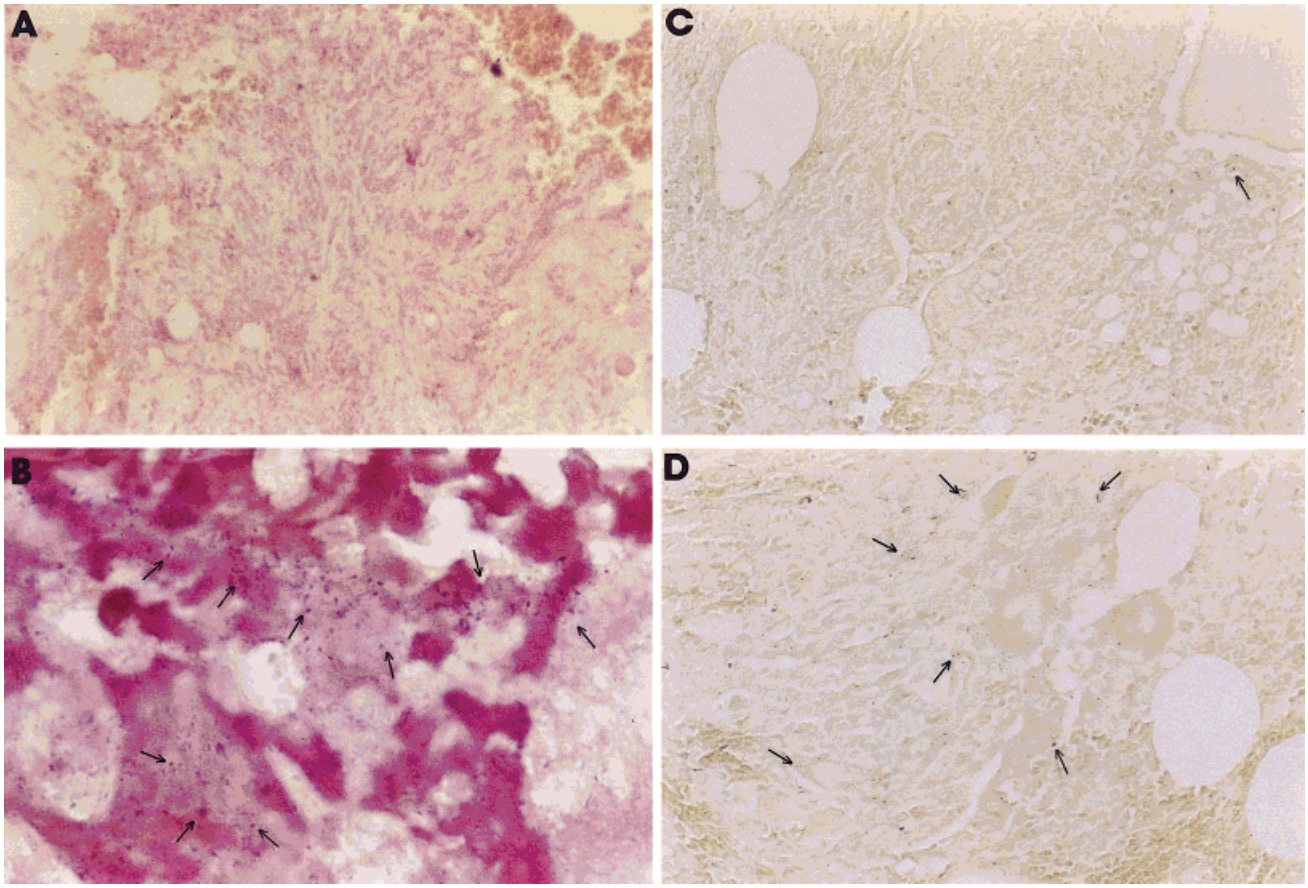

Fig. 5. In vivo detection of TGF-β1 in BM biopsies from patients with BM fibrosis. Paraffin-embedded tissues from patients with either IMF or HC were sectioned and slides that were either predominantly fibrotic (IMF: A) or hypercellular (IMF: B) were determined for TGF-β1 by in situ hybridization and immunohistochemistry (HC: C; IMF: D). Arrows indicate the areas of high density TGF-β1. Details of the techniques are described in Methods.

disorders did not have elevated TGF-β in their sera, then TGF-β levels may be useful as a potential indicator of BM fibrosis as suggested for other diseases [54]. Of further interest is a recent report that indicated that blocking biological activity of TGF-β blunts ECM protein deposits [55]. In summary, this study provides potential strategies for therapeutic intervention in patients with BM fibrosis and also for those at risk for developing this disorder. In view of a recent report that indicates a potentially useful animal model for studying IMF [56], future studies will unravel the detailed pathways leading to BM fibrosis and thus determine the particular roles of specific cytokines such as TGF-β in its pathogenesis.

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